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Biased Incorporation of Ribonucleotides on the Mitochondrial L-Strand Accounts for Apparent Strand-Asymmetric DNA Replication

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Summary

Recently, we presented evidence for conventional, strand-coupled replication of mammalian mitochondrial DNA (Holt et al. , 2000). Partially single-stranded replication intermediates detected in the same DNA preparations were assumed to derive from the previously described, strand-asymmetric mode of mitochondrial DNA replication. Here, we show that bona fide replication intermediates from highly purified mitochondria are essentially duplex throughout their length, but contain widespread regions of RNA:DNA hybrid, as a result of the incorporation of ribonucleotides on the light strand which are subsequently converted to DNA. Ribonucleotide-rich regions can be degraded to generate partially single-stranded molecules by RNase H treatment in vitro or during DNA extraction from crude mitochondria. Mammalian mitochondrial DNA replication thus proceeds mainly, or exclusively, by a strand-coupled mechanism.

Introduction

For almost 30 years, it has been known that non-replicating mammalian mitochondrial DNA (mtDNA) contains a number of ribonucleotides (Grossman et al., 1973). Standard preparations of mammalian mtDNA include apparent replicating forms that are partially single-stranded, analysis of which led to the development of the strand-asymmetric model of mammalian mtDNA replication (Clayton, 1982 and references therein). The model postulates that there are but two sites of initiation of DNA synthesis, one for each strand, denoted heavy (H) and light (L). These two origins are physically and temporally distinct. Synthesis of the H (leading) strand begins at a defined site (O_H) in the major non-coding region. Once leading strand synthesis has traversed two-thirds of the genome, the site of second strand initiation, conventionally denoted as the origin of light strand replication (O_L), is exposed on the displaced H strand. Synthesis of the lagging strand then initiates at O_L in the opposite direction. In this strand-asymmetric model of replication, DNA synthesis is continuous on both strands. Recently, conventional duplex mtDNA replication intermediates, indicative of coupled leading and lagging-strand DNA synthesis, were described in both human and mouse, apparently coexisting with partially single-stranded replication intermediates (Holt et al., 2000).

Earlier studies have failed to take into account the effect of the presence of incorporated ribonucleotides on the properties of mammalian mtDNA replication intermediates. In particular, the possibility that the excision of ribonucleotides during DNA extraction might modify the properties of such intermediates has been overlooked. Here, we demonstrate that mtDNA replication intermediates prepared from sucrose gradient-purified mitochondria are in fact almost entirely duplex. However, treatment of these preparations with RNase H yields partially single-stranded DNA molecules. Similar forms are readily detected in mtDNA extracted from cruder preparations of mitochondria. We propose that mammalian mtDNA replication proceeds principally, perhaps exclusively, by a strand-coupled mechanism, which incorporates a significant number of ribonucleotides during synthesis. Partially single-stranded "replication intermediates" observed previously, which we ascribed to the orthodox strand-asymmetric replication mode, are thus attributable to an artifact of extraction.

Results

Ribonucleotides Persist in Rat Liver mtDNA

Ribonucleotides have been reported previously to reside in mammalian mtDNA (Grossman et al., 1973), and Clayton and colleagues recognized that the numbers were significant (Brennicke and Clayton, 1981). We therefore used a combination of nucleases: RNase H, RNase One (with similar properties to RNase A), and S1 nuclease, to probe the occurrence of riboucleotides in rat liver mtDNA. A schematic map of the rat mitochondrial genome is shown in Figure 1A. RNase H treatment of rat liver mtDNA resulted in a 74% decrease in signal (Figure 1B), whereas this was not the case for plasmid DNA (pcDNA3.1, Invitrogen) (data not shown). Almost

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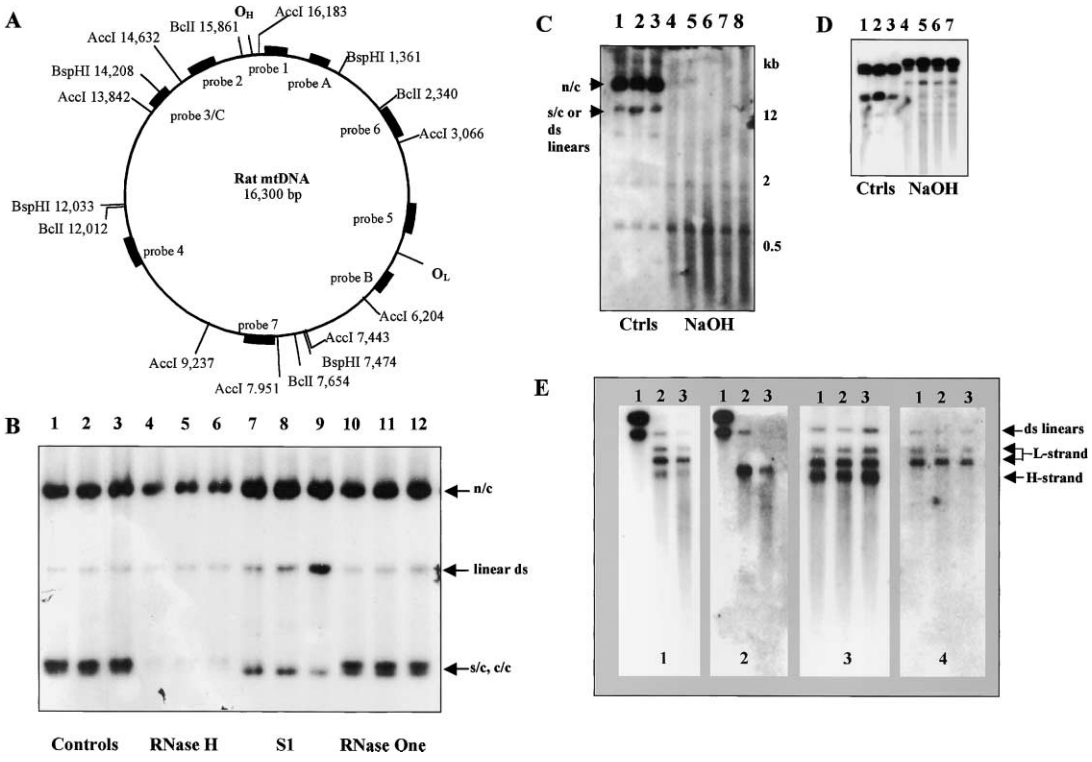

Figure 1. Non-Replicating Rat mtDNA Is Modified Markedly by RNase H, Alkali, or Heat Treatment

(A) Schematic map of the rat mitochondrial genome (Gadaleta et al., 1989), showing restriction sites for AccI, BclI and BspHI, plus the previously assigned "heavy-strand replication origin" O_H. Broad bars indicate probes that were employed to detect particular fragments of restriction digested rat mtDNA (see Experimental Procedures for details).

(B) Triplicate samples of rat liver mtDNA, derived from gradient purified mitochondria, were separated in one dimension on a 0.4% agarose gel, transferred to solid support, and hybridized to radiolabeled total rat mtDNA. Lanes 1–3, untreated mtDNA. Lanes 4–6, Rat mtDNA treated with one unit of RNase H for 60 min at 37°C. Lanes 7–9, S1 nuclease-treated mtDNA, one unit for 10 min, one unit for 60 min, and 10 units for 60 min respectively, all at 37°C. Lanes 10–12, RNase One-treated mtDNA, one unit for 60 min at 37°C. n/c – nicked circular molecules, s/c and c/c- supercoiled and presumed non-supercoiled closed-circular molecules, respectively. Essentially the same result was obtained when mtDNA from crude preparations of rat liver mitochondria were treated with these enzymes (see Experimental Procedures for details).

(C) Rat mtDNA contains alkali-sensitive sites, which are distributed throughout the molecule on both strands. Three aliquots of rat mtDNA were treated without alkali (lanes 1–3) and five aliquots were treated with 0.05 M NaOH for 1, 2, 4, 8, or 24 hr (lanes 4–8). After neutralization, samples were separated on a 0.8% agarose gel, transferred to solid support, and hybridized to probe 2. The same result was obtained when the filter was stripped and rehybridized sequentially to strand-specific forms of probe 7 (data not shown). Human placental mtDNA was similarly alkali-sensitive (see Supplemental Figure S2a available at http://www.cell.com/cgi/content/full/111/4/495/DC1), whereas a closed circular *E. coli* plasmid was not degraded after incubation with 0.05 M NaOH (D).

(E) Heat treatment of rat mtDNA. The major species generated by heat treatment were detected by probes spaced all around the genome, and both strands were represented at similar abundance, taking into account the representation of the radiolabeled nucleotide, although the intact H-strand appeared slightly more sensitive to heat than the L-strand. All lanes contain an equal amount of rat liver mtDNA extracted from gradient-purified mitochondria. E1, Lane 1, unheated control, lane 2, 98°C for 1 min, lane 3, 98°C for 5 min. The filter was hybridized with (duplex) probe 2. Heat treatments of longer than 5 min produced a smear of fragments similar to NaOH treated samples in (C) (data not shown). E2, the same filter shown in E1 was stripped and rehybridized with riboprobe C, specific for H-strand mtDNA. E3, lane 1, 98°C for 5 min; lane 3, 95°C for 5 min; lane 3, 92°C for 2 min, hybridized to (duplex) probe 2. E4: the same filter, stripped and reprobed with the version of riboprobe C specific for L-strand mtDNA. The same bands were revealed by H-strand and L-strand specific riboprobes A and B, as by riboprobe C (data not shown).

all of the remaining mtDNA migrated as open (i.e., nicked or gapped) circles. By contrast, RNase One had little effect on rat liver mtDNA. S1 nuclease, as expected, converted closed circular forms of mtDNA to open circles and subsequently to linear molecules, yet had no discernible affect on total mtDNA signal. In test experiments, it was observed that the commercial preparation of RNase H used in the study did not digest single-stranded oligonucleotides, regardless of whether or not they contained incorporated ribonucleotides. RNase H is reported to require patches of four or more ribonucleotides to initiate digestion (Roche). In further tests, RNase H removed RNA residues from an oligonucleotide containing four consecutive ribonucleotides, when base-paired to DNA, more efficiently than from a base-paired oligonucleotide containing a single ribonucleotide. In parallel experiments, RNase One was found to cleave single-stranded oligonucleotides containing either one or four incorporated ribonucleotides, but cleavage was prevented when these oligonucleotiodes were annealed

to a complementary oligonucleotide. Neither RNase H nor RNase One had a detectable effect on single-stranded oligonucleotides composed solely of deoxyribonucleotides (see Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/111/4/495/DC1).

Alkali treatment substantiated the conclusion that rat mtDNA contains numerous ribonucleotides, as it was markedly modified by sodium hydroxide treatment (Figure 1C). Incubation of rat mtDNA with alkali produced a smear of products the modal size being approximately 500 bp, suggesting that scattered ribonucleotides are distributed randomly around the genome, at least in a majority of non-replicating molecules. The same alkali treatment did not degrade control plasmid DNA (Figure 1D). Strand-specific riboprobes to each strand of the region np 7991–8404 revealed a similar pattern of degradation products (data not shown). As neither strand of mtDNA was spared, ribonucleotides are deemed to be present in both strands of rat liver mtDNA (see also Figure 1E). RNA is also highly sensitive to heat; heat treatment also led to mtDNA fragmentation. However, when the heat treatment was sufficiently brief, single-stranded species were detected (Figure 1, E2 and E4). All bands hybridized to probes from around the rat mitochondrial genome (probes 2–7) and therefore must be full or near full-genome length fragments. As each strand of non-replicating rat mtDNA had a similar propensity to fragment, the ribonucleotide content appears to be similar for the two strands. Ribonucleotides are found most frequently at intervals of approximately 500 bp, hence a typical molecule of rat liver mtDNA is likely to contain at least 30 ribonucleotides.

Junction-Containing Mitochondrial DNA Molecules, Which Are Partially Single-Stranded, Are RNase Sensitive

The first finding that led us to reexamine the issue of ribonucleotide occurrence in regard to mammalian mtDNA replication was the observation that the presumed strand-asymmetric, partially single-stranded replication intermediates (RIs) from all around the mitochondrial genome, visualized by two-dimensional agarose gel electrophoresis, were RNase One sensitive, as well as S1 nuclease sensitive in mouse (Figure 2A). The only difference between the two treatments was the appearance, after RNase One treatment, of much larger limit digestion products, estimated to be in the range 100 bp to several kilobases (Figure 2A). The same phenomenon was observed when rat mtDNA was treated with RNase One (Figure 2B). The fragments must be composed of DNA, since they were destroyed by S1 nuclease treatment either instead of (Holt et al., 2000) or subsequent to RNase One digestion (data not shown). The molecules comprising the resulting degradation arcs were judged to be single-stranded, as they coincided with restriction fragments of single-stranded M13 DNA and with arcs formed when duplex mtDNA samples were intentionally denatured and degraded by sodium hydroxide treatment (see Supplemental Figure S2B available at http://www.cell.com/cgi/content/full/111/4/495/DC1). Finally, the single-stranded fragments hybridized to strand-specific probes detecting the H-strand (Figure 2C), but failed to hybridize to equivalent probes specific for L-strand mtDNA (see Supplemental Figure S2, c and d available at above website).

Novel Slow-Moving Replication Fork Arcs of mtDNA Replication Intermediates

Two-dimensional agarose gel analysis of mtDNA RIs derived from rat liver mitochondria revealed, in addition to standard replication fork (Y) arcs, novel, slow-moving, Y-like arcs. The latter were at considerably greater relative abundance in rat liver mtDNA prepared from sucrose gradient-purified mitochondria (Figure 3B) than in rat liver mtDNA extracted from mitochondria prepared more crudely, by differential centrifugation alone (Figure 3A, see Experimental Procedures for details). From cruder mitochondrial preparations, slow-moving arcs were either a minor component or else absent altogether.

Slow-moving Y-like arcs have been reported previously in yeast mtDNA (Lockshon et al., 1995). However, those observed here differed in two respects, in that they were not perfect iterations of standard Y arcs, nor was there any accompanying recombination or X arc. On this basis, the slow-moving arcs cannot represent recombination intermediates. Rather, they appear to be products of incomplete digestion, since each slow-moving arc was detected by probes for two or more adjacent restriction fragments of mtDNA. For example, slow-moving arc *b* of BclI digested rat liver mtDNA was detected by a probe for the fragment np 12,012–15,861 (Figure 3C), but also by a probe for the adjacent fragment spanning np 15,861–2,340 (Figure 3D).

Although the slow-moving arcs contained two or more contiguous restriction fragments of mtDNA, there was no decrease in signal of the slow-moving arcs after prolonged digestion, or after digestion with increased amounts of restriction enzyme, nor did repeated phenol-chloroform extraction prior to digestion affect the integrity of slow-moving arcs (data not shown). Therefore, the failure of the restriction enzyme to cut a significant portion of sites in the RIs was not due to incomplete digestion, but must have been due to the structure of the RIs themselves. In the case of BclI, the site at np 7654 remained uncut in some mtDNA molecules, whereas that at np 2340 remained uncut in other molecules. In some molecules, both these sites resisted digestion. Only the site at 12,012 was cut in all molecules contributing to the slow-moving arcs.

The slow-moving arcs associated with BclI digests (Figure 3B and 3C) could potentially be explained by single-strand bridges linking two or more fragments, as the enzyme is unable to cleave single-stranded DNA. However, slow-moving arcs were also detected in rat liver mtDNA digested with AccI (Figures 3F, 3J, and 3I), a restriction enzyme that is able to cleave single-stranded DNA (Holt et al., 2000). As in the case of the BclI digests, each probe detected a distinct subset of these slow-moving arcs. Once again, the slow-moving arcs were absent from digests of mtDNA from crude mitochondria, where they were apparently replaced by a prominent ("sub-Y") arc of material migrating below the standard Y arc (Figure 3H). M13 single-stranded DNA, coincubated with mtDNA extracted from sucrose gradient-purified mitochondria in AccI digestions, was

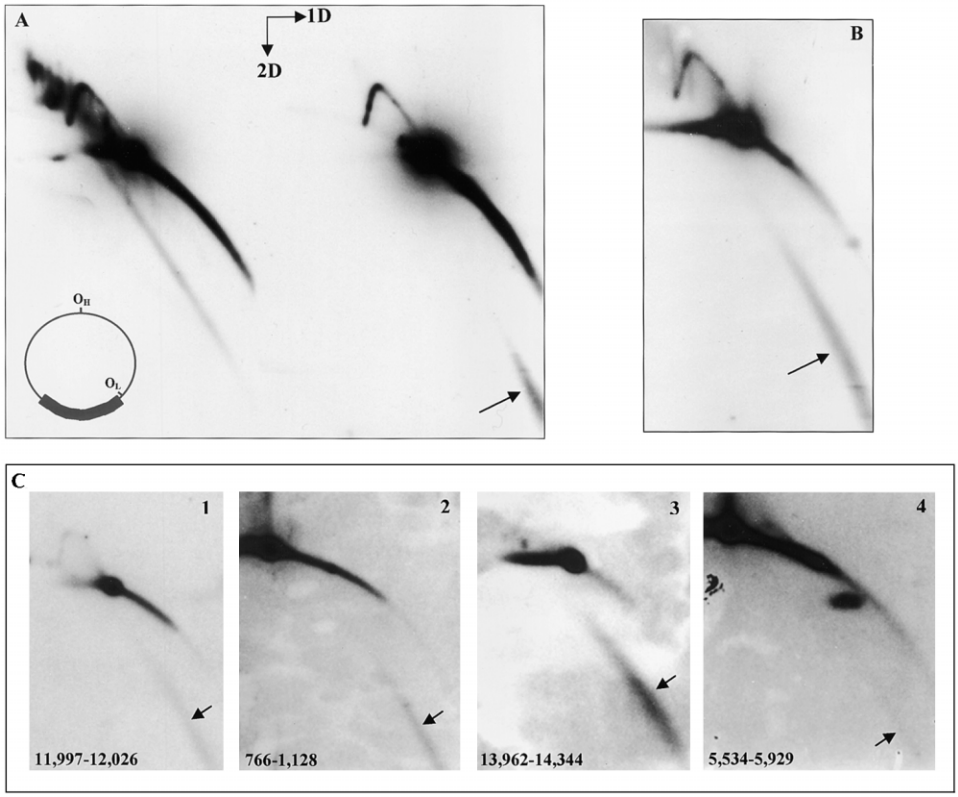

Figure 2. Partially Single-Stranded mtDNA Molecules Are Modified by RNase One as well as S1 Nuclease

Mouse liver mtDNA was restriction digested with DraI and separated on a Brewer-Fangman gel as described in Experimental Procedures. After blotting, the filter was hybridized with a probe that detected a 4.5 kb fragment of mouse mtDNA, np 5276–9817, indicated as a broad gray line on a simplified map of the mouse mitochondrial genome (A). On this and subsequent images and figures, the direction of first and second dimension electrophoresis (− to +) is as indicated by the arrows marked 1D and 2D, respectively. The two samples in (A) represent a single digest of mouse liver DNA that was divided in two, one part was incubated without enzyme in RNase One buffer (left-hand portion of the gel), the other part with RNase One, which has a similar activity to RNase A (right-hand side of A). RNase One treatment modified the partially single-stranded RIs creating a smear of fragments of lower molecular weight, indicated by an arrow, resolving the below the duplex linear arc. Rat mtDNA treated with RNase One (B and C) produced similar arcs.

(B) AccI digested mtDNA, hybridized to probe 4 (see Figure 1A). The products of RNase One digestion were single-stranded based on their mobility, which coincided with fragments of HaeIII digested single-stranded M13 DNA and denatured restriction digested rat mtDNA (data not shown).

(C) The RNase One products that resolved below the duplex linear arc were detected with strand-specific probes for the H-strand of rat mtDNA. C1, BamHI digest, oligonucleotide probe as numbered. C2 (BclI digest), and C3, C4 (BamHI digest) riboprobes, as numbered. The single-stranded arcs did not hybridize to equivalent L-strand specific probes (data not shown), and showed the same strand-bias when other restriction digests were tested with oligonucleotide probes spanning np 15,814–15,840 and 14,616–14,635 (data not shown). Note that the signal intensity varies across different regions of the genome (compare C3 and C4); an H-strand specific oligonucleotide for the region np 3052–3083 revealed almost no single-stranded arc (data not shown). No signal was detected on equivalent gels after samples had been treated with RNase-free DNase (data not shown). Mouse and rat mtDNA were derived from mitochondria prepared by differential centrifugation alone, as described previously (Holt et al., 2000).

efficiently cleaved under conditions where the slow-moving arcs remained refractory to further digestion (data not shown). Therefore, the failure to cleave must have resulted from the presence of one or more non-standard bases at the restriction site itself. As non-replicating rat mtDNA is RNase H sensitive (Figure 1B), it was inferred that the failure to cut might be due to ribonucleotides at the restriction sites.

Slow-moving arcs were detected also in mouse liver mtDNA samples purified on sucrose-gradients (Figures 3K and 3L), whereas these arcs were not detected in cruder mitochondrial preparations (Holt et al., 2000).

Slow-Moving Y-Like Arcs Are Resistant to RNase One

In crude mtDNA preparations, only the material forming a standard Y arc remained unmodified after RNase One treatment (Figure 2B and Figure 4B), whereas the slow-moving arcs associated with mtDNA from gradient-purified mitochondria were partially resistant to RNase One (Figures 4D and 4F).

Slow-moving arcs were also present in human placental mtDNA extracted from purified mitoplasts (Figure 4G), whereas they were absent from human placental mtDNA extracted from mitochondria prepared by crude

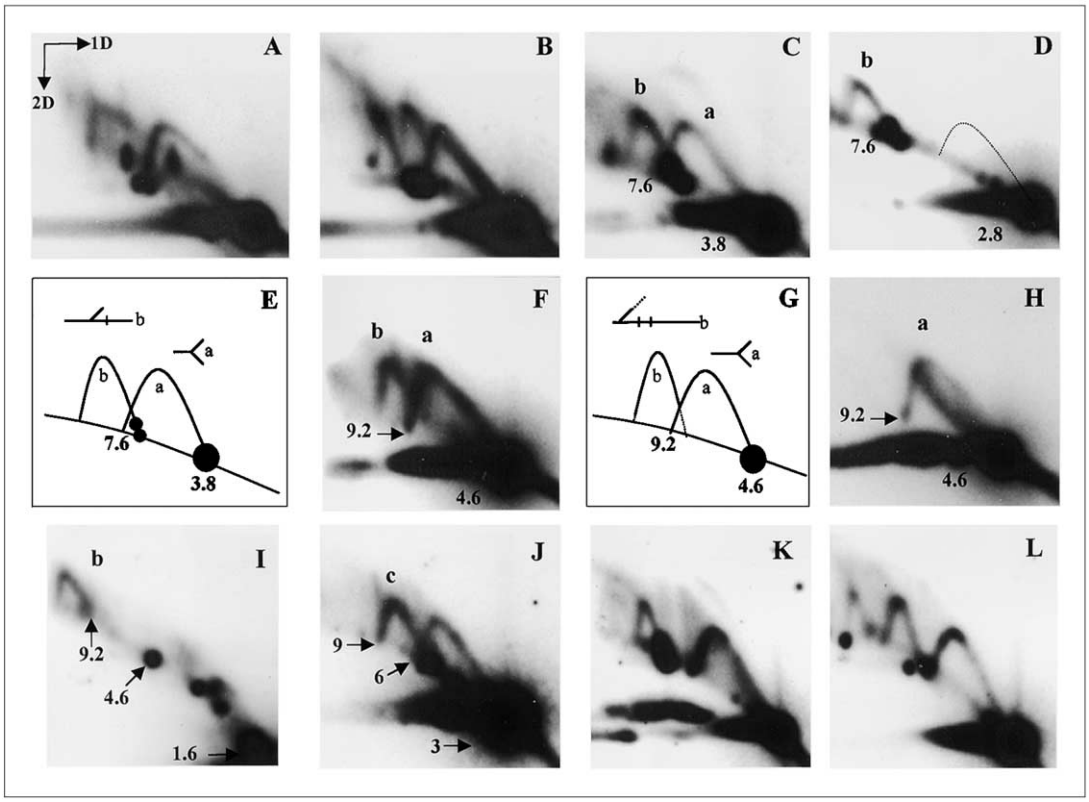

Figure 3. Novel Slow-Moving Y-Like Arcs Associated with Mammalian mtDNA

DNA was extracted from rat liver mitochondria, isolated by crude differential centrifugation (A and H), or by sucrose density-gradient purification (B–D and F) or from sucrose density-gradient purified mouse liver mitochondria (K and L). Samples in (A) and (B) were digested with BclI and hybridized to rat mtDNA probe 4, that detected the linear duplex fragment spanning nucleotides 7,654–12,012 and associated RIs. Two slow-moving, Y-like arcs were detected in mtDNA derived from gradient-purified mitochondria (B), whereas two prominent spots flanked the descending-arm of the standard Y arc in crude mtDNA samples from rat liver (A). A slow-moving arc, *b*, detected by probe 2, hybridizing to the BclI fragment of rat mtDNA spanning np 12,012–15,861 (C), was also detected by probe 1, hybridizing to the fragment spanning np 15,861–2,340 (D).

(E) shows an interpretation of the 2D gel shown in (C). Given the apparent molecular weight of the molecules of the slow-moving arc *b*, and their ability to hybridize with probes for two adjacent BclI fragments of rat mtDNA, the novel arc can be attributed to the inability of the enzyme to cut at np 15,861. A short vertical line on the sketch of the replication intermediate above the arc indicates a recognition site, where the enzyme failed to cut one branch.

(F) AccI digest of DNA from gradient-purified rat liver mitochondria, hybridized with probe 4, for the fragment spanning np 9,237–13,842. A slow-moving, Y-like arc accompanies the simple Y arc, again interpreted (G) as an inability to cleave restriction sites, in this case at np 14,632 and 13,842; see also (I) and (J).

(H) Rat liver mtDNA from crude mitochondria, when probed for the same fragment, yielded a standard Y arc and an arc beneath it, extending from the linear duplex fragment to a point close to the apex of the Y arc. Probes for other AccI fragments of rat mtDNA also detected slow-moving Y-like arcs in DNA preparations from gradient-purified mitochondria. A slow-moving arc, coincident with that seen in (F), was detected by probes 2 and 3, respectively for the fragments spanning np 14,632–16,183 (I) and 13,842–14,632 (data not shown), whereas probe 1, to AccI fragment 16,183–3,066, revealed a different slow-moving arc of lower apparent molecular weight (J). The numbers superimposed on most images are fragment sizes in kb of species on the linear duplex DNA arc.

(K and L) Sucrose-gradient purified mouse liver mtDNA digested with (K) BanII and hybridized with a probe corresponding to np 13,865–14,520 of mouse mtDNA (Bibb et al., 1981), which detected a 3.75 kb fragment spanning np 12,955–15,742, or (L) with BglI, and hybridized with a probe corresponding to np 10,577–11,215 of mouse mtDNA, which detected a 2.75 kb fragment spanning np 9,974–12,664. As with gradient-purified rat mtDNA, slow-moving, Y-like arcs were detected.

differential centrifugation (Holt et al., 2000). The slow-moving arcs of human mtDNA were, like their rat counterparts, partially resistant to RNase One treatment (Figure 4H).

Slow-Moving Y-Like Arcs Are RNase H Sensitive

Using RNase H, we tested whether the failure to cut relevant restriction sites, giving rise to slow-moving arcs, was due to the presence of ribonucleotides. Treatment with RNase H before AccI digestion essentially abolished the slow-moving arcs (Figure 5B). This was true also of restriction digests such as BspHI (Figure 5D), in which single-stranded DNA is not cut. In fact, RNase H digestion resulted in a substantial reduction in overall signal from replication intermediates, as from the unit-length fragment (Figure 5A versus Figure 5B). This implies that ribonucleotide patches are at least as frequent in replicating as in non-replicating mtDNA.

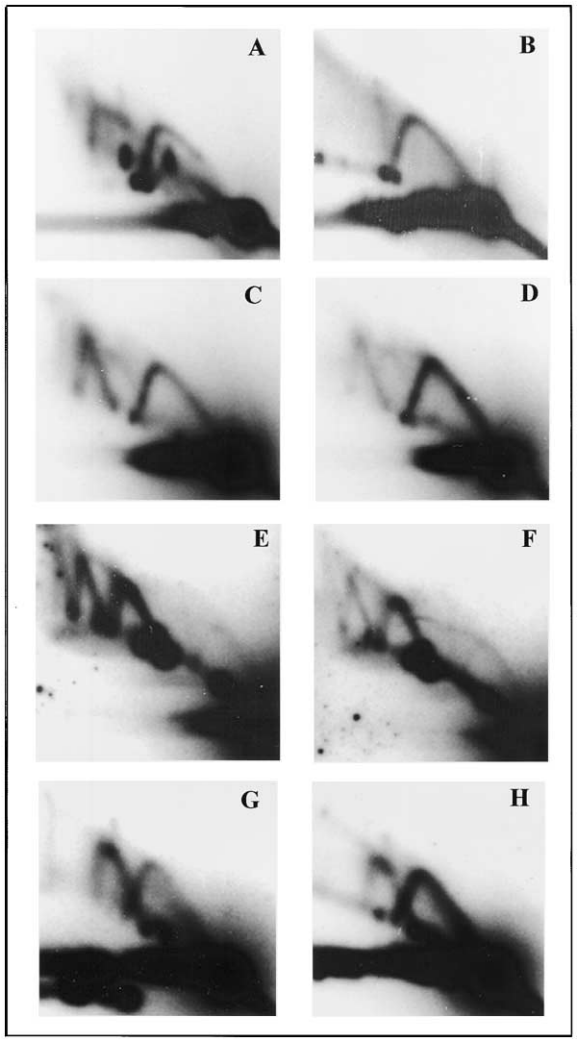

Figure 4. Slow-Moving Y-Like Arcs of mtDNA Are Partially Resistant to RNase One

A standard Y arc was the only prominent arc remaining after RNase One treatment of DNA from crude rat liver mitochondrial preparations (B versus A), whereas the slow-moving arcs seen with DNA from gradient-purified rat liver mitochondria were significantly RNase One resistant (D versus C, F versus E). The DNA samples in (A–F) were digested with BclI and hybridized to probe 4 (A–D) or probe 1 (E, F), either with (B, D, F), or without (A, C, E) RNase One. Slow-moving Y-like arcs were also seen in DNA samples from mitoplasts isolated from human placenta (G), as well as from gradient-purified mouse liver mitochondria (Figure 3K and 3L), whereas these were absent from DNA extracted from crudely prepared mitochondria of the same tissues (Holt et al., 2000).

(G and H) Human placental mitoplast mtDNA, digested with DraI, either with (G) or without (H) RNase One, hybridized with a probe for the fragment spanning np 12,271–16,010 (Anderson et al., 1981). Like their rat counterparts, slow-moving Y-like arcs of human mtDNA were significantly RNase One-resistant.

Moreover, such patches must frequently be almost coincident on the two strands, such that RNase H digestion in effect abolishes the extended duplex character of the RIs in question, degrading them to short fragments.

The slow-moving arcs were more sensitive to RNase H than standard replication fork arcs (Figure 5B and Figure 5D). Crucially, after limited RNase H digestion, the disappearance of slow-moving arcs coincided with the appearance of junctional species equivalent to those seen in crude mtDNA preparations (compare Figure 5F with Figure 3A). Note that more extensive RNase H digestion of gradient-purified mtDNA (e.g., Figure 5B), as well as RNase H digestion of crude mtDNA (Figure 6H), eliminated these sub-Y species. Sub-Y arcs were also sensitive to both S1 nuclease and RNase One (data not shown).

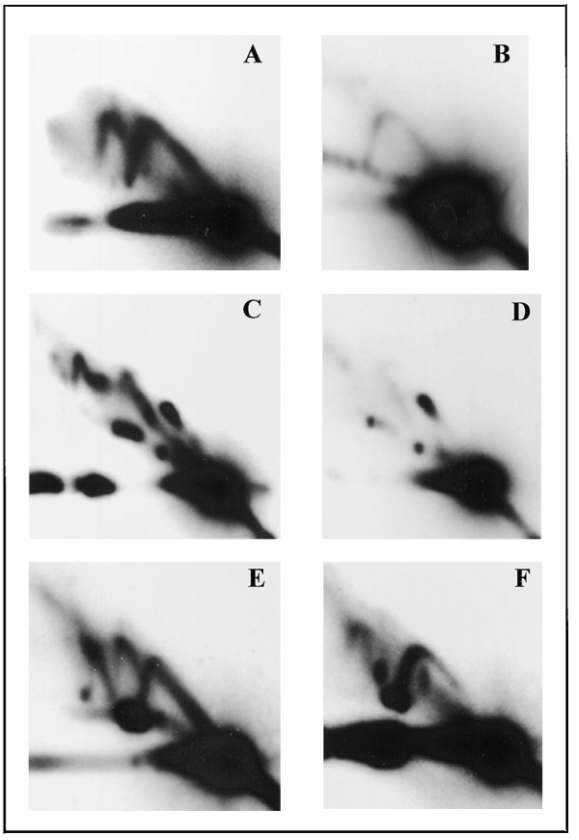

Figure 5. Slow-Moving Y-Like Arcs of Rat mtDNA Are RNase H Sensitive

Rat liver mtDNA, extracted from sucrose-gradient purified mitochondria, and digested with AccI, was treated without (A) or with one unit of RNase H for 30 min at 37°C (B). After Brewer-Fangman electrophoresis, the gel blot was hybridized with probe 4 (see Figure 1A). Exposure times were 7 and 17 hr (A and B, respectively). The two samples contained the same amount of DNA, were separated on the same gel, and hybridized together on one filter. BspHI-digested mtDNA extracted from gradient-purified mitochondria was similarly incubated without (C) or with RNase H (D), and hybridized with probe 2. The only prominent RIs resistant to RNase H were those resolving near the apex of a standard Y arc, that are presumed to result from fork arrest at O_H (Holt et al., 2000). Limited RNase H digestion of DNA samples extracted from gradient-purified mitochondria, which manifested slow-moving arcs prior to this treatment, resulted in loss of these arcs and the appearance of different molecular species (E, without RNase H and F, 0.05 units of RNase H, 20 min at 37°C). The latter were similar to those seen in DNA samples extracted from crude mitochondria (compare F with Figure 3A).

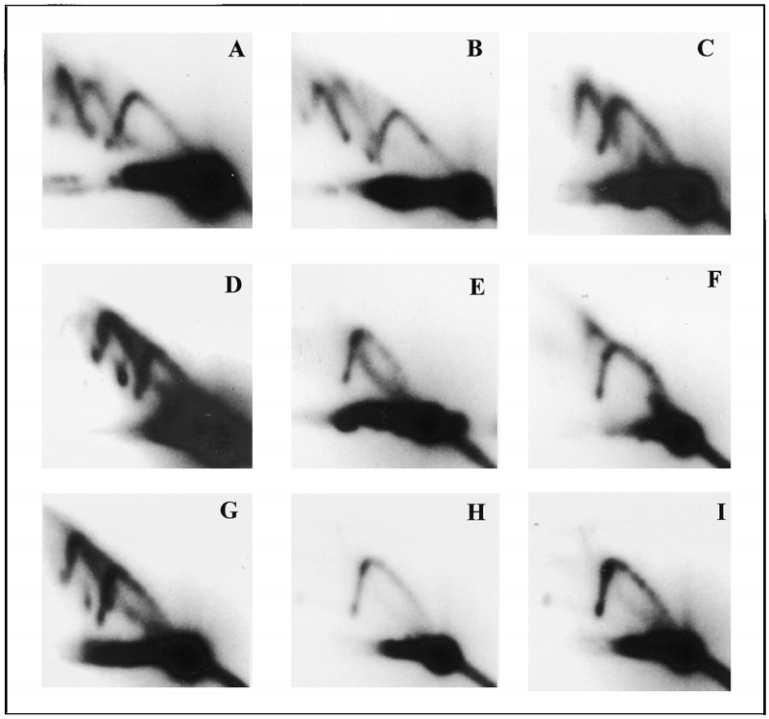

Figure 6. Effects of SSB on mtDNA Replication Intermediates

Rat liver mtDNA, prepared from gradient-purified mitochondria (A–D), or from crude mitochondria (E–I), digested with BclI (A and B) or AccI (C–I), and subjected to the following additional treatments: (A, C, E); none; (B, D, F); SSB after restriction enzyme digestion; (G); SSB prior to restriction enzyme digestion; (H); RNase H, (I): RNase H followed by SSB. Gel blots were hybridized with probe 4.

Effect of Single-Strand Binding Protein on mtDNA Replication Intermediates

As an additional test for the presence of substantial regions of single-strandedness in different RIs, we incubated rat liver mtDNA, either before or after restriction digestion, with single-stranded DNA binding protein (SSB). Slow-moving Y-like arcs were unmodified by SSB treatment of restriction digests of mtDNA prepared from gradient-purified mitochondria (Figure 6A versus Figure 6B and Figure 6C versus Figure 6D). This is consistent with the idea that the molecules comprising these arcs are substantially duplex. In contrast, SSB treatment induced a marked mobility shift in the sub-Y arc characteristic of mtDNA from crude mitochondria, which lacked slow-moving arcs (Figure 6E versus Figure 6F).

Incubation of mtDNA from crude mitochondria with SSB before rather than after restriction digestion gave a different result. Although the sub-Y arc was again diminished, slow-moving arcs appeared (e.g., Figure 6G), which were very similar to those seen in mtDNA from gradient-purified mitochondria. RNase H digestion of crude mtDNA eliminated the sub-Y arc (Figure 6H), and subsequent treatment with SSB had no further effect (Figure 6I).

Could Spontaneous RNA Hybridization to Partially Single-Stranded RIs during Extraction Account for the Findings?

Incubation of crude rat liver mtDNA with purified mitochondrial RNA failed to create slow-moving Y-like arcs, suggesting that such arcs were not the result of adventitious RNA hybridization (see Supplemental Figure S3 available at http://www.cell.com/cgi/content/full/111/4/495/DC1).

Discussion

Replicating and Non-Replicating Mitochondrial DNA Contain Incorporated Ribonucleotides

The results reported here indicate that mammalian mtDNA replication intermediates contain significantly more ribonucleotides than non-replicating mtDNA. Moreover, these ribonucleotides are prone to degradation during extraction (Figures 3 and 5), which may be due, at least in part, to relaxation and unwinding in the vicinity of the replication fork. Degradation of ribonucleotides in a region that forms an RNA-DNA hybrid will yield a partially single-stranded molecule that is sensitive to S1 nuclease digestion and can also bind SSB, i.e., will yield molecular species of the type seen in the crude mitochondrial preparations. Where ribonucleotides are preserved, novel, slow-moving Y-like arcs would be expected, as few restriction enzymes cleave RNA-DNA hybrids. Clearly, this was the case for preparations of mtDNA from highly purified mitochondria. Furthermore, limited RNase H digestion of these mtDNA samples resulted in loss of the slow-moving arcs and the appearance of molecular species similar to those seen in mtDNA from crude mitochondria.

The simplest interpretation of these experiments is that sucrose density-gradient purification of mitochondria eliminates cellular contaminants present in the cruder preparations. These contaminants include RNase H or similar nucleases that remain active during at least the first steps of nucleic acid extraction. The removal of these contaminants allows mtDNA to be extracted under conditions in which ribonucleotides remain intact in replicating molecules. The wide distribution of RNase H1 throughout the cell has recently been demonstrated by

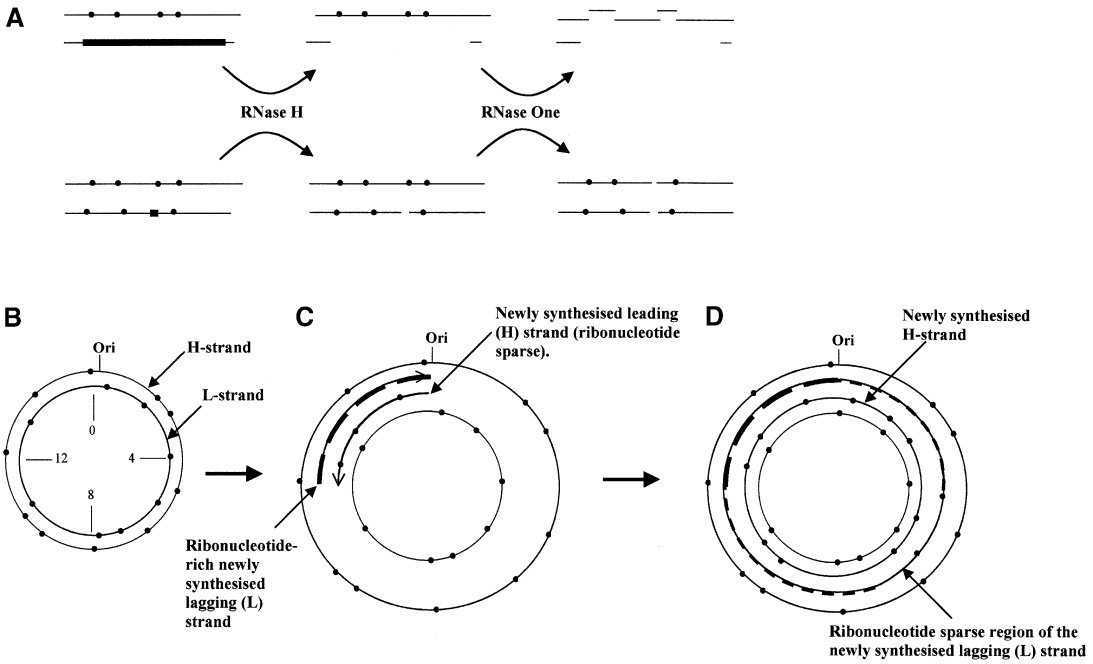

Figure 7. Ribonucleotide Distribution in Replicating and Non-Replicating Mammalian mtDNA

(A) The products of sequential digestion by RNase H and RNase One differ, according to the distribution and length of RNA patches. Isolated ribonucleotides are denoted by dots, more extended RNA patches by a bold line.

(B) The two strands of non-replicating mammalian mtDNA, represented as two circles with number scale, both contain only scattered ribonucleotides (dots). Ori-replication origin, as mapped previously (Holt et al., 2000).

(C) and (D) During mtDNA replication, the nascent H-strand contains only scattered ribonucleotides, but the nascent L-strand contains different extents of ribonucleotide content in different regions. Ribonucleotide-rich regions of the L-strand are denoted by dashed, bold lines, regions with sparser ribonucleotide content by less bold dashes, and regions with only scattered single ribonucleotides by dots, as for the H-strand.

GFP tagging in a variety of human cell types (ten Asbroek et al., 2002) and is therefore a likely source of contamination.

The slow-moving arcs are only partially resistant to RNase One (or nuclease S1), although they remain essentially unmodified by SSB. It may be inferred that even in the purest mitochondrial preparations, RIs possess limited regions of single-stranded character. In principle, this could be explained in two ways. Either the purest mitochondrial preparations that we were able to obtain are still contaminated with low levels of RNase H that remains active during extraction, or else short, single-stranded regions are actually present, e.g., close to the advancing replication fork. Such short regions should be evident even in conventional, strand-coupled RIs, since lagging-strand initiation typically occurs every few hundred base pairs.

Distribution and Abundance of Ribonucleotides in Replicating Rat mtDNA

There was no evidence of a substantial difference in ribonucleotide content between the two strands of non-replicating mtDNA, based on alkali or heat treatment (Figures 1C and 1E). However, the distribution of ribonucleotides in replicating rat mtDNA appears to be highly strand-biased, as well as non-uniform over the mitochondrial genome. After RNase One treatment of crude mtDNA samples, replication intermediates were degraded to a mixture of double- and single-stranded DNA fragments (Figure 2). Single-stranded H-strand fragments were readily detected after this treatment (Figure 2C), whereas L-strand fragments were undetectable. Moreover, some regions of the H-strand were efficiently digested to single-stranded fragments by RNase One, whereas others yielded almost exclusively double-stranded DNA fragments (compare the various images of Figure 2C). Our interpretation of this is illustrated in Figure 7.

Exposure to RNase H, as during DNA extraction from crude mitochondria, removes RNA patches longer than four nucleotides, but should leave single ribonucleotides undigested (Figure 7A). Any single ribonucleotides located on the opposite strand, in the single-stranded gaps that result, should then be digestible by RNase One, resulting in the fragmentation of the remaining strand to short DNA fragments. Conversely, in regions where ribonucleotide patches are short and infrequent on both strands, RNase H will simply introduce occasional nicks. Only those single ribonucleotides on the opposite strand that are located immediately adjacent to the sites of such nicks will be digestible by subsequent RNase One treatment, and the final product will be duplex DNA fragments of substantial length.

Thus, in non-replicating mtDNA (Figure 7B), ribonucleotide patches on both strands are short and infrequent, though nevertheless sufficient to result in fragmentation after extensive digestion with RNase H, alkali treatment, or prolonged heating (Figure 1). In replicating molecules, (Figures 7C and 7D), some portions of the lagging

(L-strand) are rich in ribonucleotides, which are inferred to occur in extended patches, spread unevenly across the genome. This interpretation is supported both by the results of RNase digestion of crude mtDNA (Figure 2) and also by the structures, RNase-sensitivity and modification by SSB of slow-moving Y-like arcs and sub-Y arcs on 2D gels (Figures 3–6). In contrast, even in replicating molecules, the H-strand appears to be largely spared from RNase digestion, giving limit single-strand DNA products in the range 100 bp to several kilobases.

Some regions of the L-strand, however, appear to be ribonucleotide sparse, even in replicating molecules. For example, the restriction sites at np 16,182, 9,236, and 6,203, (AccI), and 12,012 (BclI), were always cut, implying that ribonucleotides are never present at these positions, on either strand. Moreover, standard replication fork (Y) arcs were associated with almost all fragments analyzed; the molecules comprising a standard Y arc must contain deoxyribonucleotides on both strands at the restriction sites that delineate each fragment.

Because extensive RNA patches are absent from non-replicating mtDNA, we infer that they are removed from the newly synthesized L-strand during a maturation step. If the abundant ribonucleotides present in newly synthesized L-strand persisted, then many restriction sites of non-replicating mammalian mtDNA would be refractory to restriction enzyme digestion.

Consequences for the Validity of the Strand-Asymmetric mtDNA Replication Model

The strand-asymmetric model of mtDNA replication (Clayton, 1982) was based upon analyses of mtDNA samples that contained molecules with apparently substantial regions of single-stranded character. As documented here, such molecules are absent from mtDNA extracted from highly purified mitochondria of various mammalian tissues, yet can easily be generated during DNA extraction from cruder mitochondria. Since extensive ribonucleotide-rich regions occur only on the newly synthesized L-strand, their loss during DNA extraction would generate molecules very similar in structure to those proposed by the strand-asymmetric model. Our data do not exclude the possibility that strand-asymmetric replication may apply in some contexts, for example, in cultured cells. Nevertheless, in the tissues tested, the contribution of non-standard mechanisms of mtDNA replication must be minor.

A short D-loop, synthesized near O_H, is found in many copies of mtDNA of some tissues and cell lines, including rat liver (DeFrancesco and Attardi, 1981; Gillum and Clayton, 1978). The short single-stranded stretch of DNA can readily be detached and is also evident on Brewer-Fangman gels, where it resolves on the single-strand arc (see Supplemental Figure S2e available at http://www.cell.com/cgi/content/full/111/4/495/DC1). The significance of the short D-loop form remains unknown. There is no compelling reason to assume that the D-strand functions as a primer for strand-asymmetric DNA replication. It might act, for example, as a recruitment site for the machinery of strand-coupled replication, in the manner of initiation of ColE1 replication (Dasgupta et al., 1987). Its function may also be more associated with transcriptional regulation.

Ribonucleotides and the mtDNA Replication Machinery

The presence of significant numbers of ribonucleotides in mammalian mtDNA may be germane to its apparently prolonged replication time, estimated at approximately two hours (Bogenhagen and Clayton, 1977). This may reflect the two-stage process we have inferred, with many ribonucleotides being first introduced on the lagging-strand then subsequently replaced by deoxyribonucleotides. POLG, currently the one well-characterized DNA polymerase of mitochondria, is known to possess reverse-transcriptase activity while lacking the associated RNase H activity of viral reverse transcriptases (Kornberg and Baker, 1992). Although it appears unable to use extended, natural RNA molecules as templates (Spadari and Weissbach, 1974), the RNA-dependent DNA polymerase activity of POLG would have a clear role in allowing it to replicate DNA molecules containing scattered ribonucleotides. On the other hand, it begs the question of how the ribonucleotide patches are created in the first place. POLG, or some other mitochondrial DNA polymerase(s), must in addition possess DNA-dependent (or RNA-dependent) RNA polymerase activity to enable incorporation of ribonucleotides into mtDNA. Alternatively, relatively long mitochondrial RNAs created by transcription, RNA processing, and degradation might function as primers of lagging-strand DNA synthesis and be only inefficiently removed.

There appear to be a number of intriguing parallels between mammalian mtDNA and the prokaryotic plasmid ColE1. Like mammalian mtDNA, ColE1 is maintained and propagated as a circular molecule. It undergoes unidirectional replication (Inselburg, 1974) and also incorporates ribonucleotides under some growth conditions, e.g., when cells are grown in the presence of chloramphenicol (Blair et al., 1972). This has been suggested to arise from loss of an inherently unstable "nucleotide discrimination factor" from *E. coli* replisomes, when protein synthesis is inhibited. Further examination of this issue in *E. coli* may help elucidate how ribonucleotide incorporation into mammalian mtDNA is regulated.

Given the recognized advantages of DNA over RNA, it remains an enigma why the majority of mitochondrial DNA molecules should contain significant numbers of ribonucleotides. One possibility is that a "complete" DNA genome is in fact maintained at low abundance, perhaps in a protected environment, to serve as a master copy or mitochondrial germline. Inclusion of ribonucleotides in the chimeric genome may then be what marks it as merely a "working copy".

Experimental Procedures

Purification of Rat Liver Mitochondria

Rat liver mitochondria were isolated by a crude differential centrifugation procedure, and either processed immediately for DNA extraction or first purified further on sucrose density-gradients. Six to eight grams of rat liver was minced finely with scissors and washed three or more times with 100 ml of homogenization buffer (HB) comprising 225 mM Mannitol, 75 mM Sucrose, 10 mM Tris-HCl [pH 7.6], 1 mM EDTA, and 0.1% BSA (fatty acid free). After washing, the chopped

liver was suspended in 10 ml (per gram wet weight) of HB buffer and subjected to six strokes of Dounce homogenization with a tight fitting pestle. All operations were performed at 4°C or on ice. The homogenate was centrifuged at 1,000 g_{max} for 5 min, and the supernatant recentrifuged at 9,000 g_{max} for 10 min. The crude mitochondrial pellet was resuspended in 30–50 ml HB and the two centrifugation steps repeated. The crude mitochondrial pellet was either lysed immediately (see below) or first loaded onto a two-step sucrose gradient (1.0 M/1.5 M) and centrifuged at 40,000 g_{max} for 120 min. The final pellet was resuspended in 75 mM NaCl, 50 mM EDTA [pH 8.0], 1% SDS, 0.5 mg/ml proteinase K, and incubated either for 2 hr at 50°C or overnight at 37°C. Nucleic acid was extracted from the protease-treated mitochondrial pellet by successive incubations with phenol and chloroform, ethanol precipitated, washed and dried (Sambrook et al., 1989), resuspended in 10 mM Tris-HCl, 1 mM EDTA [pH 8.0], and stored at –20°C. Mouse and human mtDNA were isolated by the same method.

DNA Digestions

DNA prepared from mitochondria was digested with one of a number of restriction endonucleases under conditions recommended by the manufacturer (New England Biolabs). Of the enzymes used only AccI and HhaI were capable of cleaving single-stranded DNA. Where indicated in Figure Legends, 0.1–1.2 μg mtDNA was incubated with 0.05–2 units of RNase H for up to one hour at 37°C. RNase One digestion (Promega) was with five units of enzyme for 10 min at 37°C for 0.1–1.2 μg mtDNA. S1 nuclease (Promega) treatment was one unit for 2 min at 37°C. RNase H, RNase One, and S1 nuclease treatments employed buffers supplied by the manufacturer (Promega).

Alkali Treatment of Mitochondrial DNA

0.1 μg aliquots of rat mtDNA were incubated at 37°C in 0.05 M NaOH for 0.1 to 24 hr. After neutralizing with an equal volume of 1 M Tris-HCl [pH 7.6] and precipitation with ethanol, the samples were resuspended in 40 μl of 10 mM Tris, 0.1 mM EDTA, [pH 8.0], and the products separated by horizontal agarose gel electrophoresis.

SSB Treatment

Rat mtDNA, before or after restriction digestion, was coated with SSB protein from *E. coli* (Promega) at a DNA/protein mass ratio of 1:4 (wt/wt) in 1 × binding buffer (25 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, and [pH7.5]) in a total volume of 50 μl, for 30 min at 30°C. Nucleic acid-protein complexes were fixed by addition of 0.5 μl of 8% glutaraldehyde.

Two-Dimensional Agarose Gel Electrophoresis and Hybridization

Neutral/neutral two-dimensional agarose gel electrophoresis was performed by the standard method (Friedman and Brewer, 1995). First-dimension electrophoresis was at 0.7 V/cm for 20 hr in a 0.4% agarose gel without ethidium bromide (EB). After overnight electrophoresis, individual lanes of the first-dimension gel were excised and rotated through 90 degrees. A second gel of 1% agarose with 300 ng/ml EB was cast around each lane and once set the second gel was electrophoresed at 6 V/cm for 3–4 hr at 4°C. After Southern blotting (Sambrook et al., 1989), specific regions of rat mtDNA were probed for using random-primed PCR-amplified fragments. Five μl (50 μCi) of [α-^{32}P] dCTP (3000 Ci/mmol, Amersham) was incubated with three units of Klenow DNA polymerase and 50 ng of DNA, that had been annealed with 50 ng of hexadeoxyribonucleotides, for 60 min at 37°C. Oligonucleotide PCR primers (5′-3′) and the region of rat mtDNA each pair amplified are listed below. Southern hybridization was carried out in modified Church buffer (0.25 M sodium phosphate [pH 7.2], 7% SDS) overnight at 65°C. Posthybridization washes were 1 × SSC followed by 0.1 × SSC, 0.1% SDS, both for 30 min at 65°C. Filters were exposed to X-ray film and developed after 0.5–5 days.

The duplex probes used to detect fragments of rat mtDNA were generated by PCR using pairs of oligonucleotides (Sigma-Genosys) based on the published sequence (Gadaleta et al., 1989). The number assigned to each probe is as per the schematic map in Figure 1A:

Probe 1 CTAAACCCTCTTTACTTGCCTACCC and AGTTGGCACG TTTTACGCCG np 16,252-369; Probe 2 ACCTACTAGGAGACCCAG ACA and CCTGAGAAGACTGACTCTTCA, np 14,866-15,331; Probe 3 ACCCACCATAAATAGGTGAAGGC and TTTACGTCTCGGCAGAT GTGG np 13,962-14,344; Probe 4 GCCTACCCATTCATCATCCTCT and GTGTGGGAAGGTTGGAGGTT, np 10,916-11,436; Probe 5 GGA CTAGCCCCATTCCACTA and GGCTTAATAGGGCTATGATA, np 4210-4759; Probe 6 CCTCGATGTTGGATCAGGACA and AGCTAGT GTAAGGGAGAGGGT np 2416-2974; and Probe 7, GAACGCCTAAT CAGCAACCGA and CAGGCTGACTAGAAGGGTGA np 8006-8563.

In order to generate (ribo-)probes specific for the two strands of mtDNA, amplified fragments of rat mtDNA were cloned upstream of a T7 promoter, in pcDNA2.1 TOPO vector (Invitrogen) in both orientations. The letter assigned to each probe is as per the schematic map in Figure 1A:

Probe A TGAAGCGGAAAGAAATGGGC and AGTTATGTTGGTTG GTTGTAGGGC np 766-1128; Probe B GGAGGCTTCGGAAACT GACTTG and AGTGATACCTGCTGCTAATACTGGC np 5534-5929; and Probe C ACCCACCATAAATAGGTGAAGGC and TTTACGTCTC GGCAGATGTGG np 13,962-14,344.

Other synthetic oligonucleotides were used directly as probes after end-labeling:

L-strand 5′-ATCCAGCCTTTCGGTCTACTCCATTCTATGAT-3′, H-strand 5′ ATCATAGAATGGAGTAGACCGAAAGGCTGGAT-3′, np 3052-3083; L-strand 5′-TCCCTATTCGTAACATGATCAATTATA CAA-3′, H-strand 5′-TTGTATAATTGATCATGTTACGAATAGGGA-3′, np 11,997-12,026; L-strand 5′- TGAGGAGGCTTCTCAGTAGACAAA GCAACC-3′, H-strand 5′- GGTTGCTTTGTCTACTGAGAAGCCTCC TCA-3′, np 14,616-14635; and L-strand 5′-TTCCCCTTAAATAAGAC ATCTCGATGG-3′, H-strand 5′-CCATCGAGATGTCTTATTTAAGGG GAA-3′, np 15,814-15,840.

The sequences of the primer pairs used to amplify the region spanning np 10,577–11,215 and np 13,865–14,520 of mouse mtDNA (Bibb et al., 1981) were (5′-3′) AACTGAACGCCTAAACGCAGGGA plus AACTGGATTTGAAGTTGCTAGGCA, and CACAACCAACATCCCCC CTA plus GCTGTGGCTATGACTGCGAA, respectively.

The sequences of the primer pair used to amplify the region spanning np 13,285–13,802 of human mtDNA (Anderson et al., 1981) were (5′-3′) ATCGGCATCAACCAACCACACC and GTGAGTTTTAGGTAG AGGGGGATTG.

Strand-specific probes were generated by end-labeling commercial oligonucleotides (Sigma-Genosys), using five μl (50 μCi) of [γ-^{32}P] ATP (3000 Ci/mmol, Amersham) and one unit of T4 polynucleotide kinase (New England Biolabs) for 15 min at 37°C in buffer recommended by the enzyme manufacturer. The enzyme was inactivated at 60°C for 15 min and incubated with the target DNA bound to nylon membrane in modified Church buffer (see above), overnight at 42°C. Membranes were washed in three times in 3 × SSC at room temperature, before being exposed to X-ray film for one to six days at −80°C.

Radiolabeled transcripts for use as riboprobes were generated using T7 RNA polymerase. The plasmid was first linearized, then incubated with T7 RNA polymerase (Promega) in the presence of ribonucleotides, including [α-^{32}P] CTP (3000 Ci/mmol, Amersham) under conditions recommended by the enzyme manufacturer (Promega).

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